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THE ANTIGEN OF BACILLUS ANTHRACIS

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It having been shown in previous articles[1] that the antigens of many bacteria and cells consist of pure fats, it seemed advisable to inquire whether a spore-bearing organism such as B. anthracis would yield a similar antigen. For this purpose there was selected a laboratory strain of anthrax bacillus, and a strain sent me from the Hygienic Laboratory at Washington. These strains were pure, the one a meager, the other an abundant, spore producer, and were easily raised in virulence for guinea-pigs and rabbits. While the method of isolation of fatty antigens has been given before, it will be briefly summarized.

Mass cultures of the anthrax strains were grown in Roux flasks on fat-free beef peptone agar, + 0.5, at 37 C. for from 24 to 36 hours. Very heavy cultures were obtained. The growth was washed off with sufficient distilled water and transferred to large florentine flasks. To the contents of the flasks were then added KOH in slight excess and sufficient alcohol to make about 40% water-alcohol mixture. The flasks were then gently heated on water baths under reflux condensers until hydrolysis was complete as shown by clear homogeneous yellow-brown contents. The presence of unsaturated fats in anthrax being shown by the gram positive stain of the organism, further steps were carried out with the view of preventing oxidation of these fats so far as possible. The alcohol was then distilled off in vacuo and the remaining contents of the flasks concentrated to a small volume with heat under 70 C. under diminished pressure, and the fats liberated as fatty acids by acidification with 20% sulphuric acid.

After cooling, the fatty acids were shaken out with ether. The unsaponified matter was collected and again saponified with KOH-alcohol, concentrated, acidified, cooled and extracted with ether, and the process repeated until a sample of ether showed no residue on evaporation. The portions of ether were then added together, concentrated to dryness in vacuo, resaponified and then reconverted into fatty acids that were dissolved in a small quantity of ether. To this ethereal solution was then added several volumes of absolute alcohol and the whole filtered, decolorized as far as possible with animal charcoal, filtered clear, concentrated to dryness in vacuo and weighed. On the average, 200 Roux flasks of culture should yield a minimum of one gram of fatty acids, and it is desirable to obtain not less than 5 grams for purposes of observation.

Having learned from previous experience that gram-staining organisms possess unsaturated fats, and that bacteria which most readily undergo autolysis

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contain considerable quantities of soluble volatile fats, while the most resistant species appear to have their fats composed largely of the more stable, saturated, nonvolatile types, it was suspected that the anthrax bacillus would be found to contain for the most part fats of the first two types, unsaturated and volatile, because of its gram-positive character and for the reason that the organism readily autolyses at the period of sporulation. Accordingly, the fatty acids were subjected to distillation in steam until all volatile acids had passed over into the distillate. These acids were separated from the acid water by extraction with ether in the presence of solid NaCl. The ethereal solution of soluble volatile acids constituted portion A.

The remaining nonsoluble fatty acids, showing a melting point of 20-21 C., were then divided by the lead salt-ether method into portions B, a very small one consisting of saturated fatty acids, and C, containing the unsaturated fatty acids.

The acids of portion A were then converted into their methyl esters and distilled in vacuo, by which process two fractions were obtained, one distilling at 96 C. at 15 mm., the other at about 114 C. These fractions were then saponified with KOH and the respective fatty acids liberated, dissolved in ether, washed, dried, filtered and weighed. The smaller fraction was fluid at 20 C. but solidified at about 14-16 C. The neutralization value approximated 390 mg. KOH from which the calculated molecular weight would be about 150. This acid corresponded very closely with caprylic acid.

The larger portion, about 60%, or 75%, showed a melting point of 30 C., a neutralization value of 320 and a molecular weight of 170, corresponding fairly closely to capric acid. The melting point of the combined portions of A, it was stated, was 21 C. When one is dealing with a fairly narrow fraction of fatty acids, such for instance as compose portion A, it is helpful and quite permissible to draw inferences concerning the identity and proportions of the ingredients providing certain data are at hand. The mixture of volatile acids, one of which was known to be fluid at temperatures above 16 C., was regarded as consisting of about ¼ caprylic acid and ¾ capric acid before the fractional distillation was done, since a previous observation of melting points of combinations of volatile acids had recorded 20 C. as representing this particular combination. With combinations of solid, saturated acids above capric such inferences are hardly practicable since they form eutectic mixtures having often a melting point lower than either of the ingredients.

Portion B was too small to work with, but by difference it was found to make up about 5% of the total.

Portion C, a liquid fatty acid, gave a neutralization value of 200 and calculated M.W. of 280. The iodin value by the Hübl method was 88. This acid corresponded closely enough to oleic, and constituted the larger part of the total. The melting point of this acid in combination with the saturated fraction, 20 C., would indicate the saturated acid to be one lower than stearic, since the melting point of 95% oleic and 5% stearic lies above that point, and it was accordingly assumed that this small saturated fraction might consist of palmitic acid.

From this tentative analysis, then, it appeared that the fatty complex of the anthrax bacillus would be approximately represented by oleic acid 65%, palmitic acid 5%, capric acid 20% and caprylic acid 10%. In order to determine the exact constitution and proportions of the fatty acids existing as such, or as fats, or esters in the anthrax bacilli, recourse was had, as with the other antigens studied, to the delicate test of complement fixation with the serums of animals immunized with the germ bodies.

Rabbits were selected for immunization, and the fat complexes or antigens for testing were combined artificially, in varying proportions approximating that given by the analysis, from fatty acids of highest purity obtained from sources other than the germ bodies, some having been made synthetically and all being free from protein. Before being combined, the acids were converted into their sodium salts and the latter were dissolved in desired proportions in absolute alcohol in such strength that 1 c c of solution contained 2 mg. of combined salts. To each 1 c c of solution there was then added 0.8 c c of 1% alcoholic solution of purified cholesterol. These quantities and dilutions were employed because previous work had shown them to be proper for a considerable number of fatty antigens.

The complement fixation tests were carried out in a manner previously described, using as control antigen a heavy suspension of a 24-hour agar culture of anthrax bacilli in salt solution, and employing the rabbit antisheep-cell hemolytic system. Eleven antigens in all were tried, of which 6 were quickly eliminated, leaving 5 for more careful consideration, the compositions of which follow:

7. Caprylic acid 10%, capric acid 20%, oleic acid 50%.
8. Caprylic 20%, capric 20%, oleic 60%.
9. Caprylic 10%, capric 20%, oleic 55%, myristic 5%.
10. Caprylic 10%, capric 20%, oleic 65%, myristic 5%.
11. Caprylic 10%, capric 25%, oleic 60%, palmitic 5%.

The reactions of these antigens with the immune and control serums are shown in table 1.

TABLE 1

REACTION OF ANTIGENS WITH IMMUNE AND CONTROL SERUMS

Serum	Antigen 7	Antigen 8	Antigen 9	Antigen 10	Antigen 11	Anthrax Suspension	Typhoid Bacillus Suspension
Rabbit, immune 1	++	++	+	++	++	++	—
Rabbit, immune 2	+	+	++	++	++	++	—
Rabbit, immune 3	+	+	++	++	++	++	—
Rabbit, immune 4	++	++	++	+	++	++	—
Rabbit, normal	—	—	—	+	—	±	—
Rabbit, normal	—	—	+	+	±	—	—
Rabbit, normal	—	—	—	—	—	—	—
Rabbit, normal	—	—	—	±	—	—	—
Human, normal	—	—	—	—	—	—	—
Human, normal	+	±	+	+	±	±	++
Human, syphilitic	—	—	—	—	—	—	—
Human, gonorrheal	—	—	—	—	±	—	—
Antigen control	—	—	—	—	±	—	—

Antigen 11 gave reactions most closely approximating the germ suspension antigen. This antigen was then tested further for specificity by the agglutination-precipitation test described in earlier papers. Exactly the same proportions were used in the test except for the addition to the antigen of a trace of egg lecithin which was added solely for its particular colloidal properties.

Of this stabilized antigen 0.07 c c was added to 0.5 c c of salt solution in each determinant and control tube and the mixture shaken. Each tube then received 0.28 c c of the inactivated serums to be examined. After shaking again the tubes were kept in the icebox for from 8-10 hours. The precipitations are recorded in table 2.

TABLE 2

RESULT OF AGGLUTINATION-PRECIPITATION TEST

Serums	Fat—Antigens		
	Anthrax	Cholera	Typhoid
Rabbit, immune 1	++	—	—
Rabbit, immune 2	++	—	—
Rabbit, immune 3	++	—	—
Rabbit, immune 4	++	—	—
Rabbit, normal	—	—	—
Rabbit, normal	—	—	—
Human, normal	—	—	—
Human, normal	—	—	—
Antigen control	—	—	—

++ means complete precipitation, supernatant fluid clear.
— means no precipitation, homogeneous emulsion unchanged.

Artificial antigen 11 was then adopted for further work in the belief that the particular fat complex characteristic of the anthrax bacillus had been closely approximated, and the tentative proportions of the ingredients furnished by the analysis were corrected.

During the progress of observations on the antigen there developed what appeared to be, by a process of exclusion, a new means of testing its specificity, depending on the persistence of anthrax germs in the form of spores, whereby animals were made to develop spontaneous anthrax (autoinoculation) following specific lowering of resistance by injection of large doses of the artificial fat antigen. For this purpose guinea-pigs lent themselves most readily. Before taking up the experiments connected with this phase of the work it is necessary to discuss the sterility of the antigen.

During the work on the fatty antigens it has been the custom to preserve the sodium salts of the various fatty acids, freshly prepared, used in the preparation of the antigens, including anthrax, in stock solutions in absolute alcohol, from which the various complexes are prepared as needed. Repeated cultures of these solutions, and of the salts prior to their solution, have shown them to be entirely free from bacteria or spores of any kind. After combining the ingredients of the anthrax antigen, this alcoholic solution was again thoroughly tested for sterility. For fluid cultures 10 and 40 c c quantities of beef-peptone broth + 0.5, containing 0.2% glucose, were employed into which were placed graduated amounts of the alcoholic antigen from 0.01 c c (0.00001 gr.) to 5 c c. Cultures of the solution were also made on nutrient and blood agar under aerobic and anaerobic conditions. Portions of the alcoholic antigen solution were then concentrated to dryness and corresponding weights of the dry salts were subjected to culture in the same manner. All cultures were invariably sterile.

Later these tests for sterility were carefully repeated. It was found that the dose of the antigen, in 10 c c quantities of broth, which inhibited the growth of anthrax bacilli from spores, was 0.6 c c or 0.0006 mg. Cultures were then made of varying amounts of the antigen, in alcoholic solution and in powder, below the inhibiting dose, in 1 and 2 liter amounts of nutrient broth and these flasks were allowed to remain at from 35-37 C. for a month. As with the earlier tests, all cultures remained negative. After the long rest in the incubator, traces of any sediments in the flasks were then carefully pipetted off on nutrient agar. No growths occurred. Finally the antigen was filtered through a series of Berkefeld filters which were shown to be impervious to anthrax spores, and cultures of these filtrates were likewise sterile.

It is difficult to see how any germ or spore of anthrax, or of any other organism for that matter, could find its way into the antigenic materials. The absolute alcohol itself was above suspicion and the method of preparation of the antigenic powders and solutions precluded the possibility of anthrax or other contamination. As corroborative evidence of this fact, the following test was convincing: To the fatty acids entering into the antigen there were added 2 agar slants of anthrax culture 10 days old, consisting almost entirely of viable spores, and these mixtures were then subjected to the usual technic of conversion of fatty acids into the sodium salts. Of these solid salts, 50 mg. portions were inoculated into 1.5 liter quantities of nutrient broth. Of the alcoholic antigen solutions of these salts, varying amounts in duplicate were sown into 10 c c quantities of the broth. These cultures remained negative after one month's time and transplants on agar of any recoverable sediments as well.

The fatty acids themselves were of the highest purity obtainable, and were made some from vegetable sources, others synthetically. There remain to be considered the salt solution used in the dilution of the antigen for purposes of injection, the glassware, the syringes and needles, and the antigen solution ready for injection. The salt solution was prepared freshly in small amounts and autoclaved in flasks at 115 C. for 30 minutes. The pipets and other glassware were invariably sterilized in dry heat at from 200-220 C. The all-glass syringes and the needles when not in use were kept in a saturated solution of borax, and were thoroughly sterilized before using by long boiling. Cultures of the salt solution passed through the syringes, and the needles were sterile. The diluted antigen in salt solution ready for injecting was subjected, in a routine manner, to culture in broth and on agar for a long period, but no growth ever developed. In all the many hundreds of injections of numerous fatty antigens other than anthrax, similarly prepared, in animals and in man, no case of anthrax or other infection ever developed, and no case of spontaneous anthrax ever appeared in this laboratory, or in any other that the writer has learned of, until the use of the anthrax antigen was begun. It is certain then that every source of anthrax spores outside of the animals and their food has been excluded.

Exper. 1.—Four guinea-pigs, averaging 400 gm. in weight, received daily for 3 days subcutaneous injections of 0.5 mg. of artificial fatty anthrax antigen in 2 c c of salt solution. The pigs were kept in a metal-bottom, wire cage and fed on baled hay, carrots and stale bread or crackers. Two days following the last injection all the animals developed extensive subcutaneous swellings on the abdomen, became sick and died, 2 within 72 hours of the last injection, another on the 4th day and the fourth, one week after the first injection.

The necropsies all showed the same picture: Extensive subcutaneous, slightly bloody, gelatinous edema, in some areas 2 cm. thick; intestines slightly injected, the liver, and particularly the spleen, greatly enlarged, dark and friable; slight hemorrhagic exudate in the pleural cavity; lungs hemorrhagic in areas, with blood showing at the nostrils in some animals. The edema fluid contained in hanging drop fairly numerous long, large, nonmotile bacilli, sometimes single, often in short chains of 2 or more, the organisms having a definite capsule. Stained preparations from spleen fluid showed enormous masses of large gram-positive bacilli with slightly rounded free ends, but with square cut ends where joined. Cultures from the heart blood on nutrient agar under aerobic conditions at 35 C. yielded in from 12-24 hours heavy, flat, dull white ground-glass like growths which in microscopic preparations consisted of gram-positive spore-bearing bacilli. In nutrient broth the growth was less extensive, flocculent or in ropes, leaving the broth itself clear. The flocculi consisted of entwined chains of bacilli. In short, anthrax was obtained in pure culture from all animals, and the several cultures reinjected into fresh guinea-pigs and rabbits produced typical anthrax infections and death.

To determine if possible the source of the infection a similar series of 4 guinea-pigs was injected with fat antigen, and were housed in a wooden cage in an animal room in another part of the building, but were fed as before. This second series died in about the same interval of time and showed the same lesions and cultures.

A third series of 4 guinea-pigs was then selected from a large pen containing from 75-100 other pigs in still another room in the building. This series was injected as before, marked, and turned loose in the pen. All injected animals died of anthrax and yielded pure cultures, but none of the other guinea-pigs became infected.

A fourth series of 4 pigs was then injected with antigen as before and placed in an iron cage which, with its drinking vessel, had been sterilized in an oven at 200 C. for 2 hours. They were given the usual hay and other food. All the members of this series died of anthrax. These tests appeared to narrow the infection down to the food and the animals themselves.

Still another series of pigs was injected with antigen as before, confined in freshly sterilized glass jars and fed on crackers only. In all cases the water was the same as that supplied to the other animals in use in the same rooms. This series died of anthrax in 5 days, showing that probably the source of infection was within the bodies of the animals at the moment of the experiment.

Concurrently with these series of animals, similar groups of guinea-pigs were injected with diminishing doses of the fatty antigen, both subcutaneously and intraperitoneally. The results on the latter groups showed that from subcutaneous doses of ¼ of the original quantity, and from intraperitoneal doses of $\frac{1}{10}$ the amount many of the animals escaped infection, and with still lower doses the infections ceased altogether and signs of resistance to infection began to appear. It was also observed that spaced injections of the antigen intraperitoneally into guinea-pigs produced anthrax, the lowest doses being 0.01 mg. (corresponding roughly to that amount of antigenic substance contained in 6 loops of fresh anthrax culture). These facts appeared to indicate that specific sensitization varied directly with the antigenic dose.

For purposes of control the following series of guinea-pigs were used: 9 guinea-pigs, averaging 400 gm. in weight, that had received 3 subcutaneous injections of artificial gonococcus fatty antigen equal in frequency, weight and bulk to the maximum anthrax doses. Four were placed in an infected wooden cage and 4 in a wire cage in separate rooms. No animal became infected and all were well in 14 days.

Eight guinea-pigs, averaging 300 gm. in weight, received similar subcutaneous injections of artificial cholera fat antigen and were kept in infected cages for 14 days. These animals remained well.

Five guinea-pigs, 450 gm. in weight, were injected daily for 3 days with equal weight and bulk of syphilis fatty antigen and kept in infected cages. No anthrax infections resulted.

Six guinea-pigs, 400 gm. in weight, that had received intradermal inoculations of rat serum and rat anaphylatoxin and had developed Arthus' phenomenon with local necroses, were placed in cages in which guinea-pigs had died of anthrax where they remained from 2-3 weeks without signs of infection.

Six guinea-pigs that had received even larger intraperitoneal injections of the fats of tubercle bacilli and that were placed in similar surroundings did not develop anthrax.

A group of 10 pigs of from 200-225 gm. in average weight whose resistance had been impaired by intraperitoneal injections of rat serum, 4 of 5 c c, 4 of 2.5 c c and 2 of 1.5 c c were placed in pairs in ordinary glass jars and fed on the ordinary diet of hay, etc. None of this group acquired anthrax.

In a general lowering of resistance, such as accompanies Arthus' phenomenon or the intraperitoneal injection of heterologous serums into guinea-pigs, death is commonly due to an infection by a sort of septicemic organism, called pseudopneumococcus by Nicolle, to which the animals are normally susceptible. This was seen in a few instances in the present study where the doses of the anthrax and other antigens had been small and where no other infections had developed. Where, however, the animals are markedly sensitized to anthrax there is usually no time for the ordinary infection to develop. In 2 pigs injected with small doses of anthrax antigen, simultaneous anthrax and pasteurella were observed.

If the guinea-pigs in this environment are normally carriers of anthrax spores, as seems reasonable to suppose, one would expect in the lowering of specific resistance by fat antigens other than anthrax to meet occasional instances where the general lowering of resistance would give rise to spontaneous anthrax, particularly since many of the antigens contain often one or more of the same ingredients in varying proportions. Accordingly those control groups injected with gonococcus, syphilis and other antigens have been scrutinized with considerable care in the course of this work, although in none of the experiments previous to and synchronous with the use of the anthrax antigen had anthrax developed. In one instance where a series of guinea-pigs had received enormous doses of gonococcus antigen an animal died of anthrax on the 2nd day following the last injection, and one animal in a series injected with corresponding doses of syphilis antigen (sodium oleate). Undoubtedly other such cases would appear in the long run. On the other hand, it was thought too that there should be found, here and there, certain pigs free from spores at the time of injection of heavy doses of anthrax antigen that would escape the infection, and still others, spore free, in which the infection would be delayed, the ingestion of spores occurring after the injection. The suddenness of the onset and the rapid course of the disease in nearly all the animals, in some of which the infection was already under way at the time of the third injection, left no doubt regarding the presence of spores, but during repetitions of the observations on certain groups, several pigs were found in which the infection was delayed a week or more following the injections.

The supposedly different strains of anthrax bacilli picked up in this manner showed few or no morphologic or cultural variations, the chief differences being shown in broth cultures in which some strains grew in pronounced ropes, others in flocculi. In marked contrast to our old laboratory strain, all are good spore producers. None showed a virulence for guinea-pigs or rabbits greater than a single passage through an animal would account for. Many animals withstood subcutaneous doses of one or two loops of some of the strains.

Rabbits also acquire anthrax from injections of the antigen, but larger doses are required. As a rule, they are not so susceptible, and many rabbits injected with large doses escape infection. They succumb more readily to intraperitoneal doses of moderate amount spaced in 5 day intervals. Out of a series of 8 rabbits that had received 0.2 mg. of anthrax antigen in this manner for three doses, 4 died of anthrax, and 4 were shown later to be resistant to the injection of virulent culture.

Mice have not shown anthrax infection following intraperitoneal and subcutaneous injections of the anthrax antigen in doses equal to the minimum sensitizing doses for guinea-pigs.

For further purpose of control it was thought advisable to determine whether a protein derived from anthrax organisms would sensitize animals in a manner similar to the fatty antigen. For this purpose a protein was prepared and used in the following way: It was obtained from fresh 24-hour mass cultures of B. anthracis after the method described in an earlier paper.[1] After thorough extraction of the culture material with alcohol and with ether, the dry germ residue, called bacillary substance by Vaughan and Wheeler, was extracted with distilled water containing a trace of chloroform at 37 C. for 8 days, the undissolved mass removed by centrifugation at 8,000 revolutions per minute and the aqueous solution concentrated to dryness. At the same time, similar proteins were obtained from B. typhosus, and from V. cholerae. These proteins, soluble and nonheat-coagulable, and containing traces of amino acids, were fatal to rabbits in intravenous, and to guinea-pigs, of 300 gm., in intraperitoneal doses of 0.1 mg.

Eight guinea-pigs, averaging 300 gm. in weight, received 3 intraperitoneal inoculations at intervals of one day of 0.05 mg. of anthrax protein, the maximum dose consistent with safety. Following each injection the pigs were ill, but they apparently recovered within 2 days after the last injection. None of the guinea-pigs developed anthrax.

At the same time 6 rabbits were injected intravenously with the same doses and at the same intervals 2 each with typhoid protein, anthrax protein and cholera protein. Seven days after the third and last injection the 6 rabbits, together with 2 normal control rabbits, were bled from the heart, the serums separated and tested for precipitation with the various protein antigens. The serums of the normal control rabbits gave no precipitation with any antigen. The serums of the two typhoid rabbits precipitated with the typhoid, the anthrax and the cholera antigens; and the anthrax and cholera serums precipitated with their appropriate antigens and also with the 2 crossed antigens. The differences in the degrees of interprecipitation were not greater than would be accounted for by individual variations in animals. From this observation it was inferred that the 3 germ proteins prepared in this manner were probably similar in nature.

Fourteen days after the last injection—4 days after the bleeding—the 2 rabbits that had received anthrax protein were inoculated, simultaneously with 2 normal control rabbits, with the minimal fatal dose of fresh anthrax culture. All 4 rabbits died from anthrax infection within 4 days.

Exper. 2.—Having shown that probable foci of the anthrax spores exist within the bodies of the guinea-pigs and rabbits, it was thought that examination of the hay used as food would disclose the primary source.

From beneath a lifted corner of a partly used bale of hay a portion of dust was gathered from the cement floor and sifted through a flamed fine wire mesh. About 3 gm. of dust was placed in a sterile flask and shaken with 60 c c of sterile salt solution. The contents were then filtered through sterile gauze into sterile cups and centrifuged at 8,000 r. p. m. After pipetting off the clear greenish supernatant fluid, there were added to the sediment in each of two cups 2 c c of sterile salt solution. The sediment was then shaken and allowed to stand for 15 minutes to permit coarse particles to settle. From each tube one loop of fluid was passed into several tubes of melted agar and poured into plates. The balance of the fluid was injected into a series of white mice. These animals all died in from 5-8 hours from a general infection caused by a short, plump, bipolar staining, pestis-like organism. Anthrax bacilli were not found in slides or in cultures of the blood. The plates, kept at 37 C. for 24 hours yielded, among a variety of organisms, on an average one anthrax colony, though others may have been overlooked. Subcultures in broth and on agar yielded characteristic growths. One agar slant culture injected subcutaneously into 2 guinea-pigs of 400 gm., each receiving ½ slant, was sufficient to kill the animals on the 4th day. The necropsies showed typical anthrax lesions, and cultures of the heart blood in broth and on agar developed pure B. anthracis.

The second part of this experiment consisted in the examination of the contents of the stomach of a normal guinea-pig. An animal weighing 350 gm. 3 hours after feeding on baled hay, was killed by anesthesia, the stomach opened aseptically and the contents turned out into a sterile flask with a wide mouth containing 60 c c of sterile salt solution. After thorough mixing and neutralizing to litmus with sterile sodium carbonate solution, the coarser contents of the flask were allowed to settle for one hour. The supernatant cloudy fluid was pipetted into sterile cups and centrifugated at 8,000 revolutions per minute. From this point the technic was the same as in the first half of the experiment and anthrax organisms were isolated from colonies on the plates and passed through guinea-pigs.

Exper. 3.—The observations on the anthrax antigen which was passed through Berkefeld filters, shown to be impervious to anthrax spores, prior to inoculation into animals, were made the subject of a separate experiment.

Filtration of the antigen emulsified in salt solution and ready for inoculation into guinea-pigs was, of course, impossible because of its particulate nature, practically all the antigenic material remaining on the surface of the candles. It was necessary then to filter the alcoholic solution.

A number of small candles of fairly fine texture were washed with ether, alcohol and water and shown to be free from fats. They were next autoclaved at 120 C. in water for 20 minutes, and then dried and again sterilized in the oven at 200 C. Through 8 of these filters there was then passed in divided quantities of about 18 c c, 150 c c of nutrient broth, in which had been thoroughly mixed one large agar slant of 10-day culture of anthrax, containing great numbers of viable spores. The filtrates, received into separate sterile containers, were kept at from 35-37 C. for 14 days and remained sterile.

The filters were again cleaned and sterilized as above, and through each one 15 c c of the alcoholic antigen solution were then passed. From each portion there was withdrawn sufficient antigen for purposes of culture, and into 2 liter quantities of broth were inoculated 5 and 50 mg. These cultures remained sterile after 30 days at from 35-37 C. In preparing emulsions from the filtrates for injection it was observed that their character had been changed by the filtration. Nevertheless, from the filtrates of 6 filters, 12 guinea-pigs were injected subcutaneously daily for 3 days with a quantity supposed to contain 0.25 mg. of antigen, a dose equal to half that found to lead to infection in nearly all instances. None of these animals developed anthrax within 21 days.

Following the filtration of the antigen, all filters were washed with alcohol and the concentrates of the washings showed merely traces of antigen. However, on washing the filters with water in a reverse manner, the water came through milky and these aqueous portions yielded relatively large amounts of fats. It was apparent, therefore, that the antigens had been changed and had lost some of their fat content by adsorption on the filters. In order to approximate the extent and character of the changes, filters *g* and *h* of the same series of 8, after being cleaned and prepared, were tested by the passage of 0.100 gm. of antigen in alcoholic solution. Suction was maintained until the filters were perfectly dry. Through each 20 c c of absolute alcohol were then passed, and these portions added to the filtrates. The combined filtrates and alcohol washings were then quantitatively concentrated, dried and weighed. Through filter *g* there passed 0.0648 gm. Filter *h* allowed to pass 0.058 gm. Filters *g* and *h* were then washed with water in a reversed manner until the fluid came through quite clear. From the washings there were obtained of dry substance, after concentration, filter *g* 0.033 gm. and filter *h* 0.037 gm. It was therefore apparent that the filtrates used for the injection of animals were short probably at least 40% of their antigenic material. An examination of the retained substances also indicated that sodium oleate had been retained in amounts greater, proportionately, so that not only had the antigen been below strength, but its chemical composition had also been somewhat altered. The fact then that none of the 12 guinea-pigs acquired anthrax occasioned no surprise but rather was in accord with previous experience.

It was then determined to ascertain whether increasing the dosage of the filtered antigen so as to make the amount injected equal to 0.25 mg., as computed from the figures obtained above, would induce infection despite the slight alteration in the fat complex. The original filtrates of *g* and *h* were then injected in the required increased amounts and at the usual intervals into a series of 5 guinea-pigs, 2 with *g* filtrate, and 3 with *h*. Two days after the last injection, one of the animals injected with filtrate *g* died from anthrax, and cultures were obtained from the heart blood. Two days later one of the guinea-pigs injected with filtrate *h* died from anthrax infection, and yielded positive cultures from the heart blood. The other animals of the group remained well.

Exper. 4.—This experiment deals with increased resistance in rabbits against virulent anthrax organisms following immunization with artificial fatty antigen.

A recently isolated strain of B. anthracis (Hygienic Laboratory); a good spore producer, which at first was not particularly virulent for rabbits, was raised in virulence during the course of 6 months by repeated passages through these animals until an intraperitoneal dose of 0.00001 of an arbitrary 2 mm. loop was invariably fatal to full-grown rabbits in 4 days.

Eight rabbits, averaging 2,000 gm., were injected intraperitoneally 4 times at intervals of 5 days with 0.00025 gram of artificial anthrax fatty antigen suspended in 1 c c of salt solution, an amount of antigenic material contained roughly in 2 square inches of fresh culture. Five days after the last injection, 4 of the rabbits died of spontaneous anthrax, and 4 survived. The date on which the last part of the injections was given was regarded as the low point in the depression of resistance, and the surviving animals were left undisturbed for 4 weeks. At the conclusion of this time each of the 4 rabbits, together with an equal number of control rabbits, received intraperitoneally the invariably fatal dose of anthrax culture. The control animals died within 4 days, whereas the determinants remained well. These 4 surviving rabbits, after an interval of 8 days, were then injected intraperitoneally with 0.0001 of a loop of fresh virulent culture. All died from anthrax within 4 days. This showed that the rabbits to have been protected against a fatal dose of culture, but not against 100 times the fatal dose—a great excess of culture. The 4 rabbits that died from spontaneous anthrax were mentioned earlier in this paper under the experiments on specificity of antigen.

A similar group of 8 rabbits received intraperitoneally 3 injections of anthrax fatty antigen at intervals of 4 days, the first injection containing 0.0000024 grain and the following injections containing two and a half times that amount. Two of the rabbits died with snuffles 72 hours after the first injection and showed no signs of infection by anthrax. Five weeks after the last injection the 6 surviving rabbits, together with the same number of control rabbits, received the fatal dose of fresh culture. All control animals died but the determinants remained well. Eight days later these rabbits were injected with 100 times the fatal dose of anthrax culture to which they succumbed within the usual time.

A third group of 6 rabbits received 3 intraperitoneal injections of 0.00003 gm. of anthrax antigen at intervals of 14 days.

A fourth group of 6 rabbits received daily for 3 days intravenous injections of 0.00006 gm. of anthrax fatty antigen. Fifteen days after the last injections, the last two groups being timed together, the 12 rabbits, with an equal number of control normal rabbits, received intraperitoneal injections of fresh virulent anthrax culture as follows: Of group 3, two rabbits received 2 fatal doses, 2 received 4 fatal doses, and 2 received 5 fatal doses. Of group 4, two rabbits were injected with 5 fatal doses, 2 with 7 fatal doses and 2 with 10 fatal doses. All control rabbits died, and of the 2 groups all survived save one of group 4 that had received 7 fatal doses. This animal died of anthrax on the 3rd day.

As further evidence of the presence of immune bodies in the blood of these animals, the serums of the 2 rabbits that withstood 10 fatal doses of culture were tested therefor. Two normal rabbits served as controls and also 2 rabbits that had been immunized against the cholera vibrio by 4 daily intravenous injections of 0.0001 gm. of artificial cholera fatty antigen, and bled 10 days later.

The active serums of the cholera rabbits, and the same inactivated in the presence of fresh guinea-pig complement, gave strong Pfeiffer phenomena, macroscopic and microscopic, whereas the serums of the normal and the anthrax immune rabbits were negative. On the other hand, the serums of the anthrax animals alone gave positive agglutination-precipitation reactions with emulsions of anthrax antigen in salt solution.

DISCUSSION AND CONCLUSIONS

Two phases of the work require a few words of qualification. Concerning the formula for the artificial anthrax fatty antigen as published, it is understood of course that it represents antigen complex made from the purest fatty acids available to, and compounded by the writer. Those who have worked much with fats will appreciate that a label does not guarantee a pure article, and that it is often a difficult matter to obtain different lots of fatty acids all having the same values. For instance, a sample of sodium oleate (Kahlbaum) had to be discarded because the liberated fatty acid gave an iodin value of 66 instead of approximating the theoretical value of 90.07; and again, a quantity of capric acid was found to consist of a mixture of caprylic and lauric acids. The oleic acid employed had an iodin value of 87.5, but when for this acid there was substituted one having a value of 80, the antigenic properties of the mixture became somewhat impaired. It goes without saying, therefore, that were the work to be repeated by the author with entirely different lots of fatty acids there would probably occur a greater or less deviation from the antigen formula given.

The reaction of agglutination-precipitation should be regarded, like all new reactions, as tentative and in the formative stage only. The word precipitation should not be construed as having any relation to "precipitin," but rather as expressing merely the settling at the bottom of the coarse agglutinated antigen-antibody aggregate. The same factors which give rise to the specific agglutination of bacteria by antibody (agglutinins) operate in the agglutination-precipitation test. The antibody recognizes and unites specifically with the antigen whether the antigen be found at the surfaces of the bacterial cells or on the surfaces of the cholesterol in the artificial antigen emulsions, these antigenic emulsions being in a sense electro-negative suspension colloids of artificial bacteria, consisting as they do of a framework of cholesterol representing a stroma or cell surface on which is adsorbed the antigen complex.

Many of the antigen emulsions in salt solution retain the colloidal state for considerable periods of time, while others precipitate spontaneously in a short time. In the latter cases it is necessary to stabilize the emulsions in such a manner that the control tubes will not flocculate and precipitate spontaneously during the time the tubes are in the ice-box. This stability can be had either by adding a trace of purified lecithin to the alcoholic antigen solution, or by making up the salt solution in $\frac{1}{5000}$ N. NaOH solution. These substances do not impair the antigen, but tend to improve it by retarding the action of the electrolyte and the tendency to undergo hydrolysis which some complexes of alkali salts of fatty acids show sooner than others. Normal serum is of itself an excellent stabilizer.

It is an error to characterize the antigen emulsions as soap solutions, since they are not, in the first place, solutions in the strict sense of the term, and in the second place, not all alkali salts of fatty acids possess the characters of soap. Cholesterol or its equivalent is a constituent of nearly all cells and thus far has proved the best substance for the formation of particulate surfaces on which to adsorb antigenic material. It is superior to agar, starch, mastic and other higher alcohols of similar composition.

A working hypothesis for the mechanism of the reactions of immunity in the light of the facts observed during the work on antigens will form the subject of a subsequent paper.

The antigen of B. anthracis, like the antigens of many other bacteria, consists of a pure fatty complex particular and specific for that micro-organism, differing in composition from those of other bacteria studied.

The guinea-pigs and rabbits in this laboratory injected with large doses of the sterile artificial anthrax antigen, regularly develop anthrax infection, by a process of probable specific lowering of resistance, from anthrax spores of low virulence ingested with the baled hay. Small doses of the antigen raised the resistance of the animals against infection with a highly virulent strain of anthrax organisms.

Anthrax protein is not essential to the reactions and mechanism of immunity.